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Interactions of fibronectin with *Treponema pallidum*

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SUMMARY The adhesive or opsonic glycoprotein, fibronectin, is associated with the surface of *Treponema pallidum* as shown by immunofluorescence. A quantitative assay using iodine-125 (^{125}I) showed that *T pallidum* harvested seven days after infection bound more fibronectin than *T pallidum* harvested 14 days after infection. This increased binding by "younger" organisms was confirmed by radioimmunoassay techniques. Fibronectin appears to have a role in treponemal attachment. Preincubation of *T pallidum* with goat or rabbit antibody to fibronectin blocked treponemal attachment to cultured cells and to isolated capillaries and inhibited treponemal virulence. Treponemes were incubated in glass wool columns pretreated with fibronectin and were then eluted from the columns. This technique yielded a population of *T pallidum* that failed to bind to fibronectin. Compared with treponemes eluted from control ovalbumin columns, organisms eluted from fibronectin columns attached to cultured cells in larger numbers but did not survive as long and were not as virulent. Findings are discussed in terms of the relevence of interaction between treponemes and fibronectin in the pathogenesis of *T pallidum*.

Introduction

Fibronectin is an adhesive or opsonic glycoprotein that contains specific binding sites for a wide variety of substrates including collagen, fibrinogen or fibrin, actin, glycosaminoglycans, proteoglycans, DNA, plasma transglutaminase, cultured cells, and bacteria.[1-5] This glycoprotein occurs in both soluble and insoluble forms. Large amounts (300 mg/l) of soluble fibronectin are present in the blood, and smaller amounts in the cerebrospinal fluid, amniotic fluid, and saliva. Soluble fibronectin enhances macrophage phagocytosis,[6-10] and may act as a non-specific opsonin in helping to clear particulate matter from the blood. Insoluble fibronectin is a major component of basement membranes and extracellular matrices. It appears to play a part in the adhesion of cells to substrata and in cell spreading and cell morphology.[1-5]

In 1978, using heat killed formalin fixed *Staphylococcus aureus*, Kuusela first showed that fibronectin attaches to bacteria.[11] Subsequent reports have shown that fibronectin binds to many Gram positive bacteria including groups A, C, and G streptococci,[12-14] other species of staphylococci,[15] and oral streptococci.[16] Gram negative organisms such as *Escherichia coli*,[16] *Pseudomonas aeruginosa*,[17] and *Klebsiella pneumoniae*,[17] also bind very small amounts of fibronectin. In two other reports, fibronectin was shown to attach to *Candida albicans*[18] and to viral envelope glycoproteins isolated from influenza A, parainfluenza 1, and mumps viruses.[19] These studies have pointed to the potential role of fibronectin in mediating the attachment of micro-organisms to host tissues.

The attachment of pathogenic treponemes to cultured cells and non-attachment of non-pathogenic treponemes has suggested that attachment is an important part of treponemal pathogenesis.[20-22] We first reported that antibodies to fibronectin blocked treponemal attachment to cultured cells and suggested that fibronectin might be involved in treponemal attachment (Fitzgerald TJ and Repesh LA, unpublished observation, UCLA Symposium on the Molecular Biology of Host-parasite Interactions, 1983). Peterson *et al* extended this preliminary observation.[23] They found that antibodies to fibronectin blocked the attachment of treponemes to Hep 2 cells and that fibronectin reacted with three treponemal surface proteins previously implicated by Baseman and Hayes in the attachment process.[24]

We set out to investigate the relevance of interactions between treponemes and fibronectin in the pathogenesis of *T pallidum*. Within hours of entering the host, organisms "metastasise" via the blood-

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stream and lymphatic system.[25] As with disseminating tumour cells, this invasive process may be important in infecting most tissues in the secondary stage of syphilis. The mechanisms of the migration of treponemes through the interstitial matrices containing collagen I and fibronectin are poorly understood. One possibility is that there are at least two different populations of *T pallidum*. Organisms that bind fibronectin may remain localised within the extracellular matrix at the site of entry and induce the primary lesion. Organisms that do not bind fibronectin may make their way through the extracellular matrix that is rich in fibronectin, penetrate vascular channels, disseminate to other tissues, and induce secondary lesions.[26]

Materials and methods

TREPONEMES

The Nichols strain of *T pallidum* was injected intratesticularly into rabbits using 20×10^6 to 40×10^6 organisms per testis. Rabbits were given daily intramuscular injections of cortisone acetate (6 mg/kg body weight). After seven to 14 days of infection, treponemes were extracted in medium containing 90% McCoy's medium, 10% heat inactivated rabbit serum, 1 mmol/l dithiothreitol, and 30 mmol/l N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid (HEPES) pH 7·2 with a rotary shaker at room temperature for 25 minutes.[25] The suspension was centrifuged at $1000 \times g$ for 10 minutes at room temperature to sediment gross particulates. The supernatant fraction containing the organisms was then centrifuged at $12\,000 \times g$ for 30 minutes at 4°C. The pelleted treponemes were resuspended in either phosphate buffered saline (PBS) pH 7·0 or in extraction medium. Experiments in which viable organisms were incubated for various times were undertaken in an atmosphere of 2·5% oxygen, 5% carbon dioxide, and 92·5% nitrogen (which is hereafter referred to as 2·5% oxygen). In some experiments, organisms were trypsinised to remove fibronectin already bound to the surface. Treponemes were resuspended in trypsin (0·25%) and edetic acid (EDTA) (0·01%) for 10 minutes, centrifuged, washed in 10 volumes of PBS, and resuspended in PBS at a concentration of 50×10^6/ml.

BINDING RADIOIODINATED FIBRONECTIN

Commercially prepared iodine-125 (^{125}I) fibronectin was obtained from New England Nuclear (Boston, Massachusetts, USA). A volume of 50 µl treponemes in PBS at a concentration of 200×10^6/ml was added to flat bottomed wells in microtitre plates (Linbro, McLean, Virginia, USA), air dryed at room temperature, and fixed by adding 60 µl 10% ethanol and air drying.[28 29] A volume of 50 µl ^{125}I fibronectin (equivalent to 100 000 counts per minute (cpm)) was added to each well and incubated for various times at 37°C. Unbound label was removed by washing with 2·5 ml PBS, and wells were cut out and assessed for ^{125}I activity using a Beckman 4000 gamma counter. Each experiment was performed in triplicate four to six times.

IMMUNOLOGICAL DETECTION OF FIBRONECTIN

Fibronectin associated with the treponemal surface was also shown by radioimmunoassay in microtitre plates containing 96 round bottomed wells (Linbro). We used procedures identical, with one modification, to those previously described for *T pallidum*.[28 29] Briefly, 10 µl of treponemes in PBS at a concentration of 50×10^6/ml were air dried in wells, fixed with 20 µl 10% ethanol, and air dried. At this point, previous procedures used 100 µl 2% ovalbumin to minimise non-specific binding. We omitted this step as it effectively covered the fibronectin on *T pallidum*, making it non-reactive. A volume of 100 µl rabbit anti-human fibronectin (Cappel, Malvern, Pensylvania, USA, and Bethesda Research Laboratories, Maryland, USA) diluted by 1/100 was added to the test wells; the corresponding control wells received normal rabbit serum diluted by 1/100. After being left for one hour at 37°C, wells were washed to remove unbound antibody, and a volume of 100 µl ^{125}I protein A (New England Nuclear) equivalent to 100 000 cpm was added and left for a further hour at 37°C. The wells were then washed, cut out, and assessed for radioactivity. Each experiment was performed in quadruplicate on five occasions. Preliminary experiments using human fibronectin as the antigen and various dilutions of goat or rabbit antiserum indicated optimum reactivity of both antisera at a dilution of 1/100. The rabbit antiserum consistently contained more antibodies to fibronectin than the goat antiserum. In further experiments to assess the sensitivity of this assay, human fibronectin at decreasing concentrations was added as antigen, followed by a 1/100 dilution of antiserum to fibronectin. The minimum amount of fibronectin detectable using either goat or rabbit antiserum to fibronectin was 3 ng per well, which corresponded to about 200 cpm.

A second method involving immunofluorescence was used to confirm the presence of fibronectin on the surface of treponemes.[30] Treponemes were incubated for 24 hours with cultured rabbit testicular cells growing on glass coverslips.[31] Unattached organisms were removed by washing with 10 volumes of extraction medium. The coverslips were fixed in 10% methanol for 20 seconds. Rabbit antiserum to fibronectin or the control of normal rabbit serum diluted by 1/500 were added and incubated for an hour at 37°C. The preparations were washed by dipping three times in PBS and incubated with goat anti-rabbit IgG labelled with fluorescein isothiocyanate (FITC) (Miles Laboratories, Naperville, Illinois, USA) diluted 1/400 for an hour at 37°C. The coverslips were washed in PBS, a drop of 10% buffered glycerol was added, and fluorescence was observed. Maximum reactivity was recorded as 4+ and minimum reactivity as 1+. Chequerboard titrations of both the antiserum to fibronectin and the anti-IgG were performed to assess the optimum dilutions of antisera. These experiments were performed on three occasions.

BLOCKAGE OF ATTACHMENT

Treponemes suspended in extraction medium at 20 $\times 10^6$/ml were preincubated for 18 hours in 2·5% oxygen with goat or rabbit antiserum to fibronectin, or with normal goat or normal rabbit serum as controls. Various dilutions of antiserum were tested for blockage, and 1/50 was optimum. The treponemes were then added to the following substrates: human fibronectin at 100 mg/l dried on glass coverslips,[25] different types of cultured cells,[20] or intact capillaries isolated from rabbit brains.[32] After incubation for two to four hours at 37°C in 2·5% oxygen, the numbers of treponemes that were attached were compared as described previously.[21] Experiments using each substrate were performed in duplicate on five occasions.

VIRULENCE

Protocols similar to those listed for attachment were used. Treponemes (0·1 $\times 10^6$/ml) were incubated for 18 or 42 hours at 37°C in 2·5% oxygen with goat or rabbit antiserum or, as controls, normal goat or normal rabbit serum. A volume of 0·1 ml was injected intradermally into the shaved backs of Dutch Belt rabbits. A total of 24 rabbits were injected with duplicate samples. The incubation periods refer to the time between injection and initial clinical appearance of erythema and induration. Samples taken from representative lesions were observed for actively motile treponemes by dark field microscopy.

GLASS WOOL EXPERIMENTS

To deplete the treponemes that attached to fibronectin, 2 ml sterile glass wool was packed into a 3 ml syringe. The wool columns were washed with 50 ml PBS, and 2 ml human fibronectin at 100 mg/l (or 2 ml 2% ovalbumin as the control) were added to the columns and incubated for an hour at room temperature. Both columns were washed with 50 ml PBS to remove unbound fibronectin or ovalbumin. A volume of 2 ml of treponemes was then added to each column, and incubated for 0 to 120 minutes at 37°C in 2·5% oxygen. Treponemes were then eluted from the columns and counted. These experiments were repeated on three occasions.

SOURCES OF FIBRONECTIN

Affinity purified human fibronectin was obtained from Bethesda Research Laboratories or from Sigma Chemicals (St Louis, Missouri, USA). Affinity purified rabbit fibronectin was kindly provided by Dr Brett Steiner, University of Texas, Houston, USA. Stock solutions were prepared and stored at −20°C in glass tubes coated with 2% ovalbumin for 30 minutes. This coating prevented non-specific binding of the fibronectin to the storage vessel. To coat glass coverslips, 100 mg/l fibronectin was air dried in a volume of 0·3 ml of solution.[25]

Results

IMMUNOFLUORESCENCE

We used immunofluorescence techniques and antibody to fibronectin to localise fibronectin. Cultured cells were incubated with *T pallidum* and then washed to remove unattached organisms. The cultured cells that contained fibronectin provided the positive control. As with the observations of other workers,[1-5] the fibronectin on the cultured cells was distributed over the entire surface, with heavier accumulations at the edges of cells, especially at points of contact with other cells. Attached treponemes also fluoresced, which indicated fibronectin associated with the treponemal surfaces. Fluorescence was evenly distributed over the entire length of the organisms and was not localised only at the ends.

RADIOIMMUNOASSAY

In preliminary experiments, varying amounts of fibronectin were associated with the surface of treponemes using organisms harvested at different days after inoculation. To assess whether this reflected the stage of infection, organisms were harvested seven and 14 days after infection and resuspended in PBS. Portions of both treponemal preparations were trypsinised to remove endogenous fibronectin. Treponemes and trypsinised treponemes were fixed in flat bottomed microtitre wells and incubated with ^{125}I fibronectin for 0, 10, 20, 30, 40, 50, and 60 minutes. After being washed to remove unbound label, wells were cut out, and residual radioactivity was measured (figure). Treponemes harvested earlier (at day 7) bound more fibronectin that those harvested later (at day 14). In addition,

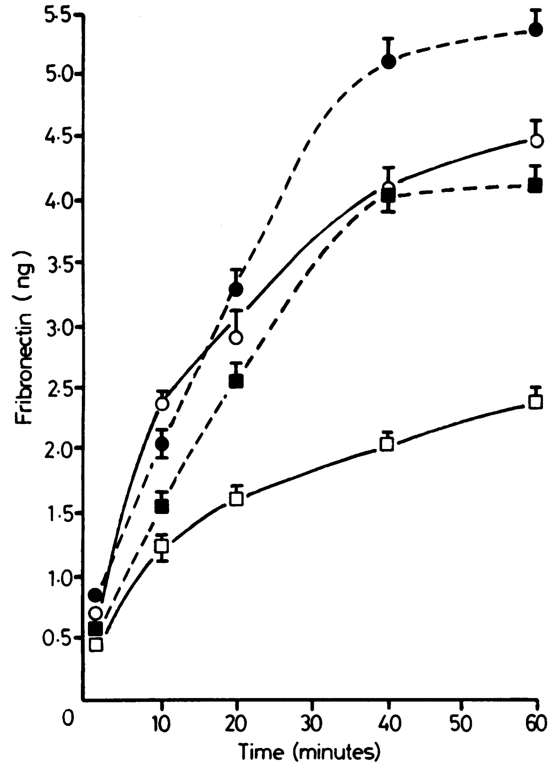

FIGURE *Residual radioactivity (expressed as ng fibronectin) at various times after adding iodine-125 to trypsinised and non-trypsinised Treponema pallidum organisms harvested at day 7 and day 14 after infection. (● = day 7 trypsinised treponemes, ○ = day 7 treponemes, ■ = day 14 trypsinised treponemes; □ = day 14 treponemes) Experiments were performed in triplicate 4-6 times; bars represent SEM.*

prior trypsinisation of organisms resulted in increased amounts of bound label. Thus less exogenous fibronectin bound to the surface of the organisms as infection progressed.

TABLE I *Results of four radioimmunoassays showing antigenic reactivity to fibronectin of treponemes harvested seven days after infection*

	Reactivity (counts per minute) of:		
Experiment	*Normal rabbit serum*	*Rabbit antiserum*	*Difference*
1	12 403	12 703	300
2	15 844	20 174	4330
3	15 285	17 203	1918
4	9 648	13 183	3535
Mean (SEM)*	13 295 (715)	15 816 (884)	2521

*Standard error of the mean for five separate experiments using triplicate samples.

Radioimmunoassay procedures were used as another way of measuring differential binding of fibronectin by treponemes. *T pallidum* was harvested from animals seven and 14 days after infection, fixed in wells, and exposed to normal serum versus antiserum to fibronectin. As table I shows, endogenous fibronectin was detected on the organisms harvested on day 7. The organisms harvested on day 14, however, failed to react with the antiserum to fibronectin, which indicated differential binding of fibronectin.

BLOCKAGE OF ATTACHMENT

In preliminary experiments antiserum to fibronectin partly blocked treponemal attachment to cultured cells. Additional experiments were performed with goat or rabbit antiserum to fibronectin using cultured rabbit testis cells, cultured rabbit dermis cells, Hep 2 cells, and isolated rabbit capillaries. Treponemes were preincubated for 18 hours with normal goat or normal rabbit serum or goat or rabbit antiserum to fibronectin. The treated organisms were then added to the three types of cultured cells, the capillaries, or glass coverslips coated with fibronectin. After being incubated for two to four hours, the attached treponemes were counted (table II). The specificity

TABLE II *Blockage of attachment of Treponema pallidum to different preparations after preincubation for 2-4 hours with goat or rabbit antiserum to fibronectin*

	Mean (SEM) No of treponemes attached after incubation with:			*% inhibition by:*	
Substrate	*Normal goat or rabbit serum*	*Goat antiserum*	*Rabbit antiserum*	*Goat antiserum*	*Rabbit antiserum*
Coverslips coated with fibronectin (treponemes/400 x magnification)	25·0 (5·2)*	4·9 (2·2)	5·2 (1·8)	80	79
Cultured rabbit testis cells (treponemes/cultured cell)	19·0 (1·9)	12·5 (1·4)	11·2 (3·3)	34	41
Cultured rabbit dermis cells (treponemes/cultured cell)	23·5 (1·0)	17·0 (1·2)	14·5 (2·4)	28	38
Cultured human Hep-2 cells (treponemes/cultured cell)	11·2 (0·7)	7·4 (1·0)	6·4 (1·3)	34	43
Isolated rabbit capillaries (treponemes/capillary)	16·5 (1·0)	11·6 (3·1)	10·0 (3·4)	30	39

*Standard error of the mean of five experiments per preparation.

of the goat and rabbit antiserum to fibronectin was indicated by the 80% and 79% blockage of attachment to the coverslips coated with fibronectin. Blockage of attachment by both antisera also occurred with each cell type and with the capillaries. The degree of blockage was consistent and ranged between 28% and 41%. We were unable to increase the degree of blockage by using longer preincubation of organisms, increased concentrations of antiserum, or decreased concentrations of *T pallidum*.

TABLE III *Virulence of treponemes after preincubation with goat or rabbit antiserum to fibronectin*

Preparation	*Mean (SEM)* days to appearance of erythema and induration*	*No (%) sites developing lesions/sites inoculated*
Normal goat/rabbit serum	18·8 (1·4)	23/24 (96)
Goat antiserum	21·7 (1·5)	19/24 (79)
Rabbit antiserum	26·3 (1·7)	18/24 (75)

*Standard error of the mean for 24 sites inoculated.

VIRULENCE

We performed related experiments to assess whether the antiserum to fibronectin would decrease treponemal virulence. *T pallidum* was incubated with normal goat or normal rabbit serum or goat or rabbit antiserum to fibronectin for 18 or 42 hours; 24 rabbits were then inoculated intradermally (table III). Virulence of *T pallidum* was not affected by the antiserum during the first 18 hours, when the incubation periods (days to the appearance of erythema and induration) and the total sites developing lesions were similar to those of the controls. Incubation for 42 hours resulted in decreased treponemal virulence. The incubation period was extended 2·9 days with goat antiserum and 7·5 days with rabbit antiserum; in addition, fewer sites developed lesions with the two anti sera. Antiserum to fibronectin therefore decreased treponemal virulence.

CROSS REACTIVITY

It was surprising that rabbit antiserum to fibronectin reacted with the fibronectin associated with treponemes that was probably of rabbit origin. We therefore used radioimmunoassay procedures to investigate whether rabbit antiserum reacted with rabbit fibronectin. *T pallidum* was trypsinised to remove endogenous fibronectin and then incubated for one hour with 100 mg/l affinity purified human fibronectin or affinity purified rabbit fibronectin. Unbound fibronectin was removed by centrifuging and washing the treponemes. The following five antigenic preparations were air dried and fixed in microtitre wells: human fibronectin, rabbit fibronectin, treponemes, treponemes previously incubated with human fibronectin, and treponemes previously incubated with rabbit fibronectin. Each set of wells was then tested with normal rabbit serum (negative control), immune rabbit serum (positive control), or rabbit antiserum to fibronectin (table IV). Compared with normal rabbit serum, immune rabbit serum gave low levels of activity in wells containing human fibronectin (762 cpm more) or rabbit fibronectin (545 cpm more), which indicated that immune rabbit serum contained antibodies to fibronectin. In the wells containing the three treponemal antigenic preparations, exposure to immune rabbit serum yielded higher levels of activity (>5300 cpm more than normal rabbit serum). This was expected as immune rabbit serum was the positive control. It also showed that the bound fibronectin did not interfere with recognition of antibody. Interesting results were obtained compared with normal rabbit serum when the five antigenic preparations were incubated with rabbit antiserum to fibronectin. This antiserum reacted strongly with human fibronectin (8711 cpm more), which was expected as rabbit antiserum to fibronectin should readily cross react with human fibronectin. The rabbit antiserum to fibronectin also reacted weakly with rabbit fibronectin (511 cpm more than with normal rabbit serum). Similarly the treponemes previously incubated with human fibronectin reacted strongly, and those previously incubated with rabbit fibronectin reacted weakly. These results show that rabbit antiserum to fibronectin reacts with rabbit fibronectin.

TABLE IV *Results of radioimmunoassays showing reactivity of rabbit antiserum to fibronectin with rabbit fibronectin*

	Mean (SEM) counts per minute of:*				
Antigenic preparation coating plastic wells	*Normal rabbit serum*	*Immune rabbit serum*	*Net difference*	*Rabbit antiserum*	*Net difference*
Human fibronectin	1324 (264)	2086 (495)	762	10035 (1690)	8711
Rabbit fibronectin	1947 (404)	2492 (508)	545	2458 (493)	511
Treponemes	2066 (295)	7403 (1205)	5337	2377 (432)	311
Treponemes previously incubated with human fibronectin	2270 (324)	7661 (1206)	5391	8075 (1466)	5805
Treponemes previously incubated with rabbit fibronectin	1803 (338)	7496 (1235)	5693	2334 (451)	531

*Standard error of the mean of three separate experiments using triplicate samples.

TABLE V *Attachment of treponemes to glass wool treated with fibronectin or ovalbumin*

	Ovalbumin glass wool		*Fibronectin glass wool*	
Time (min)	*Mean (SEM)* treponemes eluted (× 10^7/ml)*	*% decrease*	*Mean (SEM)* treponemes eluted (× 10^7/ml)*	*% decrease*
0	3·17 (0·6)	0	3·17 (0·6)	0
30	3·17 (0·2)	0	2·17 (0·7)	32
60	2·83 (0·2)	11	2·08 (0·5)	34
120	2·92 (0·6)	8	1·58 (0·2)	50

*Standard error of the mean of three separate experiments using duplicate samples.

GLASS WOOL EXPERIMENTS

The data in table II indicate that goat or rabbit antiserum partly, but not totally, blocked treponemal attachment to cultured cells (28% to 41% blockage). The inability to attain better blockage may reflect different populations of *T pallidum*, some of which do not bind to cultured cells through fibronectin and are therefore not affected by antiserum to fibronectin. To show different populations, treponemes from day 7 infections were incubated in glass wool columns precoated with fibronectin or (as a control) ovalbumin. After incubation for 0 to 120 minutes, samples were eluted from each column and treponemes were counted (table V). After the first 30 minutes, 32% of the organisms were retained in the fibronectin column compared with none in the ovalbumin column. After 120 minutes, 50% of the organisms were retained in the fibronectin column compared with 8% in the ovalbumin column. When these experiments were repeated using *T pallidum* from day 14 infections, few treponemes were retained in the fibronectin column. This agrees with the observations in table I, the figure, and table II, in which the day 14 treponemes bound fibronectin or reacted poorly with antiserum to fibronectin.

The glass wool technique at least partly depleted the population of treponemes that bind fibronectin. In the last series of experiments, attempts were made to identify differences in this population. Organisms were added to columns treated with ovalbumin or fibronectin. After 30 minutes treponemes were eluted from both columns and adjusted to equal concentrations. Both preparations were then added to coverslips coated with fibronectin (to indicate specificity of depletion) or to cultured cells (to assess treponemal attachment and survival), or were immediately inoculated intradermally into rabbits (to assess virulence). Table VI shows the differences between the two preparations of *T pallidum*. Using organisms eluted from the fibronectin column, 70% fewer treponemes were attached to coverslips coated with fibronectin, which indicated partial depletion of the organisms binding to fibronectin; whereas larger numbers attached to the cultured cells but did not survive as long. In addition, this population of *T pallidum* exhibited longer incubation periods in vivo, which indicated differences in virulence. These three variables show differences in the two populations of treponemes.

Discussion

Our earlier studies pointed to reactivity of fibronectin with *T pallidum*. Organisms adhered to glass coverslips coated with fibronectin, and IgG from serum immune to syphilis blocked that attachment.[25] In addition, attachment of treponemes to cultured cells was partly blocked by pretreatment of organisms with antibodies to fibronectin (Fitzgerald TJ, Repesh LA, unpublished observation, UCLA Symposium on the Molecular Biology of Host-parasite Interactions, 1983). The findings reported

TABLE VI *Attachment, survival, and virulence of treponemes eluted from glass wool treated with ovalbumin or fibronectin*

	Ovalbumin glass wool	*fibronectin glass wool*
Treponemes incubated for 10 hours on coverslips coated with fibronectin:		
Mean (SEM)* organisms/400 × magnification	70·7 (2·5)	21·2 (2·1)
Treponemes incubated for six hours with cultured cells:		
Mean (SEM)* attached/cell	21·3 (3·5)	36·3 (4·3)
Mean (SEM)* hours to 50% motility	68·2 (16·3)	29·6 (3·8)
Treponemes immediately injected intradermally into rabbits:		
Mean (SEM)* days to appearance of erythema and induration	8·8 (0·9)	12·6 (1·1)
No of sites developing lesions/sites inoculated	30/32	31/32

*Standard error of the mean of four experiments using duplicate samples.

here confirm and extend these earlier studies. The interaction of *T pallidum* with fibronectin was shown by binding assays using soluble ^{125}I human fibronectin. Prior trypsinisation of organisms resulted in increased binding of fibronectin either by removing endogenous fibronectin (which was sensitive to trypsin) or by exposing additional receptors or ligands of fibronectin on the surface of treponemes. The presence of fibronectin on the surface of *T pallidum* was also shown immunologically by immunofluorescence and radioimmunoassay and by blocked attachment and decreased virulence after preincubation with antiserum to fibronectin.

The binding of fibronectin to *T pallidum* appears to be different from the binding of fibronectin to cultured cells or other constituents. In unpublished observations treponemes were incubated with ^{125}I fibronectin and then centrifuged at high speed to pellet the organisms. This washing procedure removed most of the bound ^{125}I activity. If fibronectin possessed a specific binding site for *T pallidum,* high speed centrifuging should not disrupt the complex of treponemes and fibronectin. Fibronectin does have a binding site for glycosaminoglycans. *T pallidum* contains glycosaminoglycans associated with its surface, which are effectively removed by high speed centrifuging.[33] Fibronectin may bind to these treponemal glycosaminoglycans, which, with their bound fibronectin, would be stripped away by high speed centrifuging. This would explain the relatively weak binding of fibronectin to *T pallidum*.

Fibronectin binds to other micro-organisms and has been implicated in attachment to different tissues. Myhre and Kuusela[13] and Switkalski *et al*[12] showed that groups A, C, and G streptococci bound fibronectin. Further work presented evidence of two separate binding sites for staphylococci and streptococci.[34] Simpson and Beachey suggested that fibronectin mediated attachment of group A streptococci to oral epithelial cells through streptococcal lipoteichoic acid.[14] Espersen and Clemmensen identified a staphylococcal surface protein that bound fibronectin.[15] Babu *et al* suggested that cariogenic streptococci attached to dental tissues through the fibronectin present in the pellicle of teeth.[16] Abraham *et al* found two different populations of human buccal epithelial cells, one that was heavily coated with fibronectin and one that was not; *Streptococcus pyogenes* attached only to the buccal cells that contained fibronectin.[35] In other studies fibronectin has been shown to bind to *Salmonella typhimurium, Bacillus subtilis, Saccharamyces cerevisiae, Candida albicans,* and viral glycoproteins.[19 36] Fibronectin was first thought to react with Gram positive but not Gram negative bacteria.[35] Recent studies, however, have indicated that Gram negative bacteria also bind fibronectin, but bind far less than Gram positive bacteria. The quantity of fibronectin bound by *T pallidum* was quite similar to that reported for *Escherichia coli* (20 ng/10^9 cells), *Pseudomonas aeruginosa* (10 ng/10^9 cells), and *Klebsiella pneumoniae* (7 ng/10^9 cells).

Baseman and Hayes identified three putative treponemal proteins that mediate attachment of *T pallidum* to Hep 2 cells,[24] and fibronectin has recently been reported to attach to each of these proteins.[23] Preincubation of Hep 2 cells with antiserum to fibronectin has been shown to result in 84% blockage of treponemal attachment, which led to the conclusion that attachment of *T pallidum* to host tissues resulted solely from interactions with fibronectin. We disagree with this explanation for two reasons. Firstly, it is unlikely in view of the clinical manifestations of syphilis. When *T pallidum* first enters the host, some of the organisms remain localised in the extracellular matrix, multiply, and induce the primary lesion, whereas other organisms disseminate to other tissues within hours via the bloodstream and lymphatic system. If all the treponemes bound fibronectin, they would be unable to penetrate through the extracellular matrix, which is rich in fibronectin, to reach the vascular channels and disseminate. In addition, findings in this report and that of Peterson *et al* showed that *T pallidum* binds soluble fibronectin.[23] Once organisms enter the circulation, therefore, the large excess (300 mg/l) of soluble fibronectin in the blood would rapidly saturate the fibronectin ligands of the treponemes. If fibronectin ligands are solely responsible for tissue attachment, the organisms with saturated ligands would not be able to attach to other host tissues at sites distant from the primary lesions and induce secondary lesions. Secondly, preincubation of *T pallidum* with antiserum to fibronectin resulted in partial but not total blockage of attachment (28% to 41% blockage). Various different experimental conditions were tested, but we were unsuccessful in increasing the degree of blockage. Thus some of the organisms apparently attach to cultured cells through mechanisms other than fibronectin. We recently published findings on the ability of *T pallidum* to attach to laminin, collagens, and glycosaminoglycans,[25] each of which may be present in different types of cultured cells. Treponemal attachment to these cellular components would not be affected by antibody to fibronectin.

The mechanisms of treponemal attachment to cultured cells are probably quite complex. *T pallidum* apparently possesses receptors or ligands for fibronectin that could bind direct to the insoluble fibronectin on the surfaces of cultured cells. *T pallidum* also binds soluble fibronectin that is present in both inflamed tissues[37] and the bloodstream. Fibronectin has binding sites for a wide variety of tissue components (collagens, laminin, and hyaluronic acid) in addition to a binding site for itself. The soluble fibronectin already associated with the treponemal surface could therefore, in turn, bind to the collagen, laminin, hyaluronic acid, and insoluble fibronectin within tissues. Lastly, *T pallidum* coats itself with hyaluronic acid as infection progresses[33]; treponemal associated hyaluronic acid could bind to the insoluble tissue fibronectin.

The data in Table VI suggest that there are different populations of *T pallidum*, some of which do not bind fibronectin. For this reason, preincubation with antiserum to fibronectin would not be expected to totally block treponemal attachment to cultured cells. The glass wool experiments indicated that organisms that did not bind fibronectin showed differences in attachment, survival, and virulence. In addition, day 7 treponemes bound more fibronectin and reacted far better with antiserum to fibronectin than day 14 treponemes.

As a biological precedent for different populations of *T pallidum*, strains within species have been shown to bind varying amounts of fibronectin. Switalski *et al* showed that 13 of 17 strains of *Streptococcus hominis*, 11 of 20 strains of *Staphylococcus saprophyticus*, and four of seven strains of *Staphylococcus epidermidis* bound fibronectin.[12] Simpson *et al* reported variation within seven strains of *Streptococcus pyogenes* with a range of 63 ng to 1089 ng fibronectin/10^9 bacteria.[17] Espersen and Clemmensen observed variation in binding of fibronectin by different isolates of *Staphylococcus aureus*.[15] Myhre and Kuusela found wide variations in fibronectin binding using 387 strains belonging to 35 different species of microorganisms.[13] Thus it seems likely that *T pallidum* may vary in its ability to bind fibronectin. Experiments are currently in progress to test the hypothesis that treponemes that fail to bind fibronectin are responsible for disseminated syphilitic infection.

Finally, we were surprised to observe the reactivity of rabbit antiserum to fibronectin with fibronectin associated with treponemes. As there are no reports of biosynthesis of fibronectin by micro-organisms, it is reasonable to assume that the fibronectin on the surface of *T pallidum* is of rabbit origin. In general, antiserum to fibronectin does not react with fibronectin isolated from the same species. We initially intended to show that goat antiserum to fibronectin would block treponemal attachment, as a positive control, and rabbit antiserum would not, as a negative control. Our data indicated, however, that rabbit antiserum reacted with treponemes in radioimmunoassay, blocked treponemal attachment to cultured cells, and reduced treponemal virulence. The observations in table IV showed that rabbit antiserum to fibronectin did recognise isolated rabbit fibronectin or rabbit fibronectin associated with *T pallidum*. This confirmed the reports of Murphy-Ullrich *et al*, who showed that denaturation of mouse fibronectin resulted in reactivity with mouse antiserum to fibronectin.[38] The binding of rabbit fibronectin to the surface of treponemes may sufficiently alter the conformation of rabbit fibronectin to render it immunogenic in the rabbit system. This would explain the presence of antibodies to fibronectin in serum immune to syphilis (table IV). We have also detected antibodies to laminin in serum from animals with syphilis (unpublished findings). As a biological precedent, antibodies to laminin develop in Chagas disease.[39,40] It was suggested that *Trypanasoma cruzi* incorporated the patient's laminin on to its surface, which modified its structure to render it immunogenic. These antibodies, in turn, may contribute to tissue histopathology through an autoimmune like reaction. Similarly, Murphy-Ullrich *et al* reported autoimmune mediated injury to the glomerular basement membranes in mice immunised with denatured mouse fibronectin or laminin, and in rabbits immunised with human fibronectin.[38,41,42] In syphilis, it has been proposed that autoimmune like phenomena contribute to histopathology of lesions; this is especially well documented in the nephrotic syndrome in secondary syphilis. The antibodies to fibronectin and laminin could partly explain host mediated damage within lesions of syphilis.

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